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Molecular and Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry-Based Characterization of Clinically Significant Melanized Fungi in India

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ABSTRACT Melanized or black fungi are a heterogeneous group of fungi causing cutaneous to systemic diseases with high mortality. These fungi are rarely reported as agents of human infections, primarily due to difficulties in their classical identification. In this study, we examined, using molecular methods and matrix-assisted laser desorption ioniazation–time of flight mass spectrometry (MALDI-TOF MS), the diversity of melanized fungi (MF) isolated from patients in 19 medical centers in India. Overall, during a 4-year period, 718 (5.3%) clinical specimens yielded MF. Of these, 72 (10%) isolates had clinical significance and were identified primarily by sequencing the internal transcribed spacer and large subunit (LSU) regions. MF represented 21 genera comprising 29 species, the majority of them belonging to the orders Pleosporales (50%) and Chaetothyriales (22%). Among the 29 fungal species identified in this study, only 6 (20%) species were identified by the MALDI-TOF MS due to the limited commercial database of Bruker Daltonics for MF. However, a 100% identification rate of 20 additional species identified in this study was obtained by constructing an in-house database using 24- to 96-h-old liquid cultures. Further, the CLSI broth microdilution method revealed low MICs for posaconazole (≤1 μg/ml) and voriconazole (≤2 μg/ml) in 96% and 95% of isolates, respectively. Skin/subcutaneous and sinonasal and pulmonary phaeohyphomycosis due to MF were diagnosed in 21.4% (n = 15) and 28.5% (n = 20) of cases. Also, 10% of patients had central nervous system involvement (n = 7), and 3 cases of fungal osteomyelitis due to *Cladophialophora bantiana* and *Corynespora* spp. were observed.

KEYWORDS melanized fungi, phaeohyphomycosis, MALDI-TOF MS, antifungal resistance, India

Melanized or black fungi are considered to be rare etiologic agents of human infections worldwide, primarily attributed to difficulties in their classical identification (1). Although these fungi are ubiquitously distributed and are often isolated in microbiology laboratories, their clinical significance is not yet entirely elucidated. Consequently, infections caused by melanized fungi (MF) are frequently underestimated, and clinical experience with these fungi is limited. A study from the MD Anderson Cancer Center, Texas, assessed the range of clinical presentations due to infections caused by MF in cancer patients during 1998 to 2008 and reported that only

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11% of MF isolates had clinical significance. Notably, these MF isolates exhibited resistance to antifungal drugs (2). Also, a population-based active laboratory surveillance study for invasive mycotic infections in a large San Francisco Bay metropolitan area, conducted during 1992 and 1993, reported one case/million persons/year due to MF (3). A more recent Transplant-Associated Infection Surveillance Network study in 23 U.S. centers from 2001 to 2006 reported that cumulatively, zygomycosis, fusariosis, phaeohyphomycosis (PHM), and other molds constituted ~10% of cases (4). PHM, which was originally described by Ajello (5), includes a heterogeneous group of fungal infections whose etiologic agents develop in host tissue as dark-walled, melanized septate mycelial elements. The other end of the spectrum of disease caused by MF is represented by chromoblastomycosis (CMB), which is a progressive disorder limited to the skin and subcutaneous tissue with the characteristic microscopic finding of muriform cells (1, 6).

In recent years, with the introduction of molecular diagnostics, infections caused by the MF are being diagnosed increasingly among both healthy as well as immunocompromised hosts. More than 130 species from 70 genera have been recorded to be associated with infections in humans and animals (6). The fungal diseases caused by these agents are of serious concern because MF can infect and kill apparently healthy individuals. The spectrum of clinical infections caused by these fungi is broad, ranging from allergic respiratory manifestations, keratitis, and subcutaneous involvement to systemic diseases involving lung, brain, and paranasal sinuses (1, 7, 8). The accurate identification of MF is important, because different species may vary with regard to tropism to the lungs, brain, or other organs and susceptibility to antifungal agents (9). In recent years, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has emerged as a rapid and accurate identification tool for filamentous molds (10–14). However, with regard to black fungi, studies analyzing MALDI-TOF MS for their identification and database creation are limited (10–12). Here, we examined, using molecular methods and MALDI-TOF MS, the diversity of MF isolated from patients with suspected fungal infections from 19 medical centers in India during a 4-year period. In addition, their clinical significance and antifungal drug susceptibility profiles were determined.

RESULTS

The details of 72 isolates obtained from 70 patients analyzed in the present study are described in Table 1. They originated from skin biopsy specimens ($n = 14$), corneal scrapings ($n = 15$), sputa ($n = 10$), bronchiolar lavage (BAL)/bronchial aspirates ($n = 10$), fine-needle aspiration biopsy (FNAB) lung specimens ($n = 5$), brain abscesses ($n = 7$), nasal tissues ($n = 5$), tissue biopsy specimens ($n = 4$), blood ($n = 1$), and pus ($n = 1$).

ITS, D1/D2, and *β-tubulin* gene sequencing and phenotypic identification. Of the 72 isolates, both internal transcribed spacer (ITS) and large subunit (LSU) region sequencing identified 89% ($n = 64$) of isolates up to the species level, and a single isolate of *Cladophialophora carrionii* was identified by *β-tubulin* gene sequencing. The remaining 7 isolates were assigned to genus-level identification. Overall, equivalent species-level identification was achieved by both ITS (70.8%, $n = 51$) and LSU (69.4%, $n = 50$) region sequencing. However, genus-level identification of only 26% ($n = 19$) was obtained by LSU region sequencing. About 29% ($n = 21$) and 5% ($n = 3$) of the isolates repeatedly yielded no amplification for ITS and D1/D2 regions, respectively. A total of 22 genera comprising 29 species were identified in this study using the above-described markers, and the majority of the species identified belonged to the order Pleosporales (50%, $n = 36$), followed by Chaetothyriales (22%, $n = 16$) (Table 1). Overall, using phenotypic methods, morphologically only 40% ($n = 30$) of the isolates that showed typical sporulation characteristics were identified up to the species level. However, 35% ($n = 24$) of the isolates were placed in the respective genus. Notably, 25% ($n = 18$) of isolates remained unidentified by morphological methods. The ITS

TABLE 1 Species distribution and clinical details of melanized fungi (n = 72)

Order (no. of isolates)	Species identified[a] (n)	GenBank accession no.		Diagnosis (strain, no.)	Specimen type (n)
		ITS	D1/D2		
Pleosporales (36)	*Medicopsis romeroi** (3), *Exserohilum rostratum** (4), *Alternaria alternata* (9), *Bipolaris cynodontis** (3), *Curvularia lunata* (6), *Curvularia hawaiiensis** (3), *Curvularia clavata* (1), *Curvularia kusanoi** (1), *Curvularia verruculosa** (1), *Curvularia geniculata** (1), *Curvularia* spp. (1), *Roussoella percutanea* (1), *Phoma multirostrata* (1), *Corynespora* spp. (1)	KU361135, KY310616–KY310625, JN880415, KY310626–KY310633, FR717533, KY310634–KY310636	KU361119, KY303834–KY303868	Subcutaneous phaeohyphomycosis (*M. romeroi*, 3; *A. alternata*, 2) osteomyelitis (*Corynespora* spp., 1); allergic bronchopulmonary mycosis (*A. alternata*, 6; *C. lunata*, 2; *C. verruculosa*, 1; *C. hawaiiensis*, 3); allergic fungal rhinosinusitis (*E. rostratum*, 2; *A. alternata*, 1); keratitis (*E. rostratum*, 2; *C. lunata*, 4; *C. geniculata*, 1; *Curvularia* spp., 1; *R. percutanea*, 1; *P. multirostrata*, 1); colonizer (*B. cynodontis*, 3; *C. clavata*, 1; *C. kusanoi*, 1)	Sputum (10), corneal scrapings (10), BAL fluid (2), bronchial aspirate (2), FNAB (n = 3), skin biopsy specimen (5), nasal tissue (3), tissue biopsy specimen (1)
Chaetothyriales (16)	*Fonsecaea monophora** (3), *Cladophialophora bantiana** (6), *Cladophialophora carrionii* (1), *Exophiala dermatitidis* (3), *Exophiala jeanselmei** (1), *Exophiala spinifera** (1), *Rhinocladiella mackenziei** (1)	KY310637, KY310638, KM225276, KY310639–KY310650	KM225277, KY303869–KY303876, KY273520, KY303877–KY303881	Subcutaneous phaeohyphomycosis (*C. carrionii*, 1; *E. jeanselmei*, 1; *E. dermatitidis*, 1; *E. spinifera*, 1); chromoblastomycosis (*F. monophora*, 2); brain abscess (*C. bantiana*, 4; *R. mackenziei*, 1; *F. monophora*, 1); pulmonary phaeohyphomycosis (*E. dermatitidis*, 2); osteomyelitis (*C. bantiana*, 2)	Brain abscess/tissue (6), skin biopsy specimen (6), BAL fluid (2), tissue biopsy specimen (2)
Hysteriales (3)	*Rhytidhysteron* spp.* (3)	KY310651–KY310653	KY303882–KY303884	Subcutaneous phaeohyphomycosis (*Rhytidhysteron* spp., 2); chromoblastomycosis (*Rhytidhysteron* spp., 1)	Skin biopsy specimen (3)
Sordariales (8)	*Cryptendoxyla hypophloia** (2), *Chaetomium* spp. (2), *Chaetomium globosum* (1), *Phialemonium dimorphosprum** (2), *Madurella mycetomatis** (1)	KY310659, KY310661, KY310662	KY303890–KY303897	Pulmonary phaeohyphomycosis (*C. globosum*, 1); keratitis (*P. dimorphosporum*, 1; *Chaetomium* spp., 2); eumycetoma (*M. mycetomatis*, 1); colonizer (*P. dimorphosporum*, 1; *C. hypophloia*, 2)	Corneal scrapings (3), BAL fluid (1), FNAB (2), bronchial aspirate (1), tissue biopsy specimen (1)
Capnodiales (3)	*Cladosporium cladosporioides* (3)	KY310654–KY310656	KY303885–KY303887	Brain abscess (*C. cladosporioides*, 1); fungemia (*C. cladosporioides*, 2)	Brain tissue (1), blood (1), bronchial aspirate (1)
Botryosphaeriales (2)	*Lasiodiplodia theobromae** (1), *Lasiodiplodia parva** (1)	KY310657, KY310658	KY303888	Keratitis (*L. theobromae*, 1); postoperative sepsis and dissemination (*L. parva*, 1)	Pus (1), corneal scrapings (1)
Microascales (2)	*Ceratocystis adiposa** (1), *Thielaviopsis ethacetica** (1)	KF060719, KY310660	KF060720, KY303889	Allergic fungal rhinosinusitis (*C. adiposa*, 1; *T. ethacetica*, 1)	Nasal tissue (2)
Incertae sedis (2)	*Acrophialophora fusispora** (2)	KY310663, KY310664	KY303898, KY303899	Keratitis (*A. fusispora*, 2)	Corneal scrapings (2)

[a]An asterisk denotes that an in-house MSP database was created for MALDI-TOF MS and validated against the CBS reference strains (*M. romeroi* CBS 252.60, *B. cynodontis* CBS 109894, *C. hawaiiensis* CBS 173.57, *C. verruculosa* CBS 150.63, *E. rostratum* CBS 134641, *F. monophora* CBS 102248, *C. bantiana* CBS 173.52, *E. jeanselmei* CBS 507.90, *E. spinifera* CBS 899.68, *R. mackenziei* CBS 650.93, *Rhytidhysteron* sp. strain FMR 8743, *M. mycetomatis* CBS 109801, *C. hypophloia* CBS 508.70, *P. dimorphosporum* CBS 491.82, *L. theobromae* CBS 124.13, *C. adiposa* CBS 138.34, *A. fusispora* CBS 380.55).

phylogenetic neighbor-joining (NJ) tree (Fig. 1) of MF yielded distinct clades representing 8 orders, along with the reference/type strains ($n = 23$) retrieved from GenBank.

MALDI-TOF MS. The current updated (September 2016) Bruker Biotyper OC, version 3.1, database contain main spectra (MSP) of 25 species of MF, representing 16 genera. Among 29 fungal species identified in this study, only 6 (20%) species, namely, *Alternaria alternata*, *Curvularia clavata*, *Curvularia lunata*, *Exophiala dermatitidis*, *Cladosporium cladosporioides*, and *Chaetomium globosum*, were available in the current database and identified by the MALDI-TOF MS with a score of ≥2. Also, a single isolate of *P. dimorphosporum* was identified as *Phialemonium* spp. with a score of ≥2 due to a lack of species data of this genus in the database. Of 29 species identified in the present study, 6 species that were already available in the database and additional 3 species, *viz*., *Roussoella percutanea*, *Phoma multirostrata*, and *C. carrionii*, were excluded for in-house database creation. Therefore, the in-house database created for 20 species identified all of the MF isolated as well as CBS (Table 1) and Vallabhbhai Patel Chest Institute (VPCI culture collection) reference strains of respective species with a score of ≥2. In concordance with the ITS tree, the MSP dendrogram (data not shown) also showed order-wise clustering of the isolates.

Antifungal susceptibility testing. The results of *in vitro* antifungal susceptibility profiles of 56 isolates tested are summarized in Table 2. Among the azoles, posaconazole (POS) was the most active drug (MIC range, 0.015 to 8 μg/ml) for the MF except *Lasiodiplodia parva* and *Lasiodiplodia theobromae*, which showed MICs of >8 μg/ml. Also, itraconazole (ITC; MIC range, 0.03 to 16 μg/ml) and isavuconazole (ISA; MIC range, 0.015 to 8 μg/ml) showed low MICs for the isolates tested. However, single isolates of *C. lunata*, *Curvularia verruculosa*, *R. percutanea*, and two species of *Lasiodiplodia* each exhibited high MICs of ITC (MIC range, 2 to 16 μg/ml). Also, MICs of ≥2 μg/ml for ISA were noted for *A. alternata*, *Exserohilum rostratum*, *C. verruculosa*, and *Exophiala jeanselmei*. Voriconazole (VRC) MICs of ≥2 μg/ml were observed for all 4 isolates of *E. rostratum* and single isolates of *Bipolaris cynodontis*, *C. lunata*, and *R. percutanea*. Amphotericin B (AMB) and echinocandins showed variable MIC and minimum effective concentrations (MECs), respectively, against MF. High AMB MICs (MIC range, 4 to 8 μg/ml) were recorded against *E. jeanselmei*, *Rhinocladiella mackenziei*, *Thielaviopsis ethacetica*, and single isolates of *Cladophialophora bantiana* and *C. cladosporioides*. Notably, echinocandins were not active (MEC range, 2 to 8 μg/ml) against *E. dermatitidis*, *E. jeanselmei*, *R. mackenziei*, *T. ethacetica*, *R. percutanea*, and *Madurella mycetomatis* and single isolates of *A. alternata*, *C. bantiana*, and *C. cladosporioides*.

Clinical conditions, underlying risk factors, and treatment outcomes of patients. The clinical and laboratory records of the 70 patients showed that two important clinical entities were mainly diagnosed in these patient. The first group had skin and subcutaneous infections ($n = 15$), which included 11 cases of subcutaneous PHM, 3 cases of CMB, and a solitary case of mycetoma (Fig. 2). The other large group comprised sino-nasal and pulmonary mycosis ($n = 20$), which included 12 cases of allergic bronchopulmonary mycoses (ABPM), 5 of allergic fungal rhinosinusitis (AFRS), and 3 of pulmonary PHM. Further, significant numbers of patients had central nervous system (CNS) involvement ($n = 7$) and osteomyelitis ($n = 3$). Also, in this series 15 cases of keratitis were diagnosed. Fungemia and postoperative sepsis with dissemination was observed in one case each. In the remaining 8 cases the significance of MF isolation from respiratory specimens could not be attributed to pulmonary disease and probably represented colonization in other underlying lung diseases. In subcutaneous PHM, no risk factors were found in 50% of cases. However, in 3 subcutaneous PHM due to *Medicopsis romeroi*, two had a renal transplant and one patient had lepromatous leprosy with diabetes mellitus (type II). In the remaining 3 subcutaneous PHM cases, history of trauma and peripheral vascular diseases on long-term steroids (Fig. 2A) was recorded. Among the three cases of CMB, two cases had a renal transplant and were on immunosuppressive drugs; one of the patients also was on antitubercular drugs due to abdominal tuberculosis. However, trauma to the foot with a wooden splinter was noted

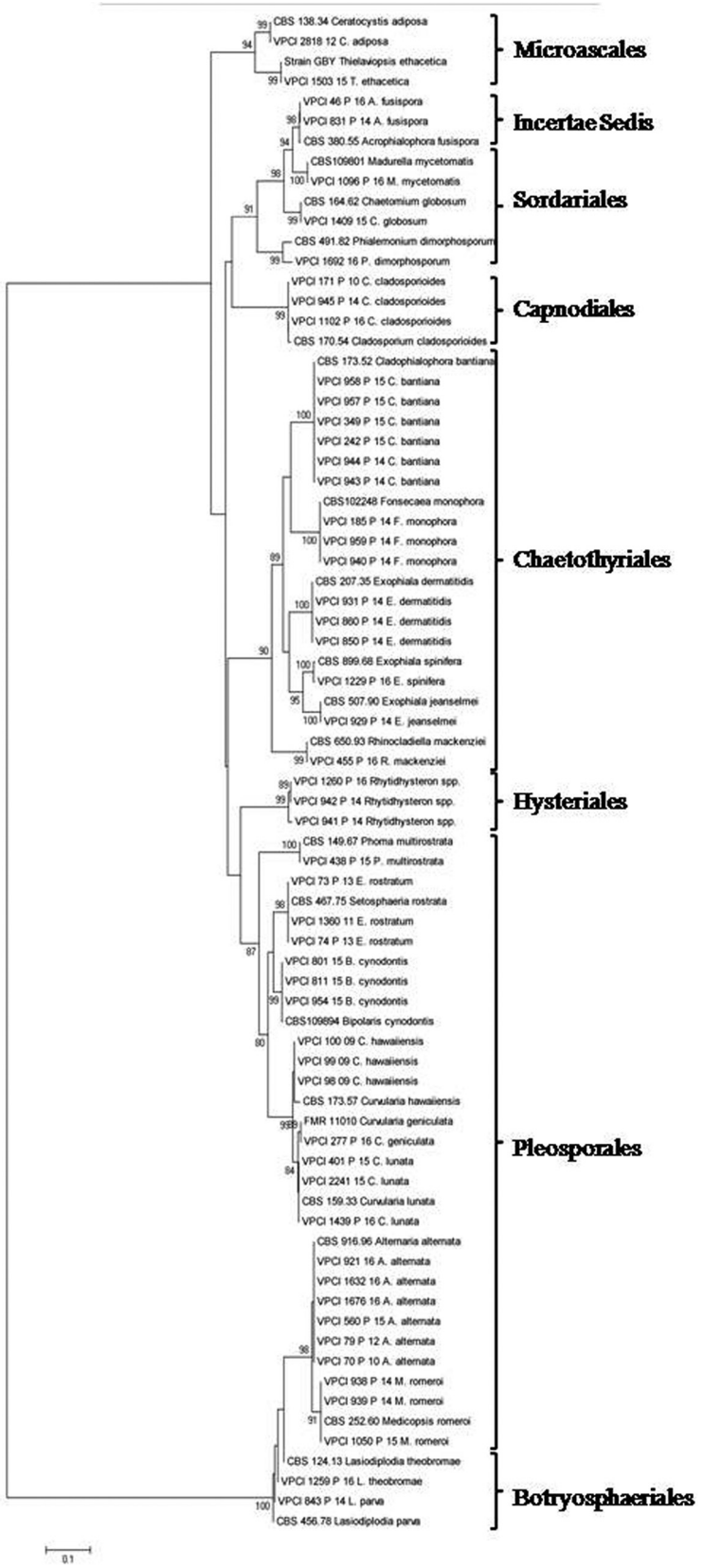

FIG 1 Phylogenetic tree based on ITS sequences using neighbor-joining analysis, using MEGA version 6, with 2,000 bootstrap replications of melanized fungi. Sequences of Indian strains (VPCI numbers) along with type/reference strains ($n = 23$) were retrieved from GenBank for the analysis. Bootstrap values are shown above the branches.

TABLE 2 *In vitro* antifungal susceptibility profile of 56 melanized fungi against antifungal agents

Order and species (no. of isolates)	Drugs[a]	MIC[b]/MEC[c] range (μg/ml)	No. of isolates with MIC/MEC (μg/ml) of:											
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64
Pleosporales (30)															
Medicopsis romeroi (3)	AMB	0.125–1				1	1		1						
	VRC	0.125–0.25				2	1								
	ISA	0.015	3												
	POS	0.03–0.06		1	2										
	ITC	0.06–1			1		1		1						
	FLU	64													3
	CAS	8										3			
	MFG	8										3			
	AFG	8										3			
	5-FC	4–8									2	1			
Exserohilum rostratum (4)	AMB	0.125–0.25				2	2								
	VRC	1–4							1	2	1				
	ISA	1–2							1	3					
	POS	0.06–0.25			1	2	1								
	ITC	0.125–0.5				2	1	1							
	FLU	16–64											1	2	1
	CAS	0.5–1						3	1						
	MFG	0.06–0.25			2	1	1								
	AFG	0.03–0.25		1	1	1	1								
	5-FC	8–64										1		2	1
Alternaria alternata (9)	AMB	0.03–2		1		1	2		3	2					
	VRC	0.125–1				1	1	1	6						
	ISA	0.25–4					1	1	2	3	2				
	POS	0.015–1	3		3	1			2						
	ITC	0.125–0.5				3	4	2							
	FLU	2–64								2			2	1	4
	CAS	0.015–8	1			1	3	2	1			1			
	MFG	0.015–8	1	2	1	2	2					1			
	AFG	0.015–8	4		1	2		1				1			
	5-FC	32–64												2	7
Bipolaris cynodontis (3)	AMB	0.125–0.25				2	1								
	VRC	1–2							2	1					
	ISA	0.125–0.25				1	2								
	POS	0.125–0.5				1	1	1							
	ITC	0.125–0.25				2	1								
	FLU	16–32											1	2	
	CAS	0.5–1						2	1						
	MFG	0.125–0.5				1		2							
	AFG	0.25–1					1	1	1						
	5-FC	32–64												1	2
Curvularia lunata (6)	AMB	0.125–1				1	2	2	1						
	VRC	0.25–4					2	3			1				
	ISA	0.06–0.5			3	2		1							
	POS	0.06–0.5			1	2	2	1							
	ITC	0.125–8				1	1	3				1			
	FLU	2–64								1			2	2	1
	CAS	0.25–2					2	3		1					
	MFG	0.06–1			2	1	2		1						
	AFG	0.06–1			1	3		1	1						
	5-FC	64													6
Curvularia hawaiiensis (3)	AMB	0.03–0.125		1	1	1									
	VRC	0.25–1					1	1	1						
	ITC	0.03–0.06		2	1										
	CAS	0.5						3							
	MFG	0.125				3									
	AFG	0.125				3									
Curvularia verruculosa (1)	AMB	0.25					1								
	VRC	1							1						
	ISA	2								1					
	POS	0.06			1										
	ITC	4									1				

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TABLE 2 (Continued)

Order and species (no. of isolates)	Drugs[a]	MIC[b]/MEC[c] range (μg/ml)	No. of isolates with MIC/MEC (μg/ml) of:												
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64
	FLU	64													1
	CAS	0.5						1							
	MFG	0.5						1							
	AFG	0.5						1							
	5-FC	64													1
Roussoella percutanea (1)	AMB	0.5						1							
	VRC	4									1				
	ISA	0.5						1							
	POS	1							1						
	ITC	2								1					
	FLU	64													1
	CAS	4									1				
	MFG	8										1			
	AFG	8										1			
	5-FC	64													1
Chaetothyriales (15)															
Fonsecaea monophora (3)	AMB	0.5–1						1	2						
	VRC	0.125–0.5				1	1	1							
	ISA	0.015–0.25	1		1		1								
	POS	0.015–0.06	1		2										
	ITC	0.06–0.125			2	1									
	FLU	8–64										1	1		1
	CAS	0.125–0.5				1		2							
	MFG	0.25–0.5					2	1							
	AFG	0.125–1				1	1		1						
	5-FC	32–64												2	1
Cladophialophora bantiana (6)	AMB	0.125–8				1				4		1			
	VRC	0.06–1			1		1	3	1						
	ISA	0.015–0.25	1			3	2								
	POS	0.015–0.125	2		1	3									
	ITC	0.03–0.25		2		3	1								
	FLU	2–64								1		1	3		1
	CAS	0.125–8				1			2	2		1			
	MFG	0.125–8				1		1	2	1		1			
	AFG	0.125–8				1			3	1		1			
	5-FC	0.125–1				2	1	2	1						
Cladophialophora carrionii (1)	AMB	1							1						
	VRC	0.5						1							
	ISA	1							1						
	POS	0.25					1								
	ITC	0.5						1							
	FLU	64													1
	CAS	0.25					1								
	MFG	0.5						1							
	AFG	0.25					1								
	5-FC	64													1
Exophiala dermatitidis (3)	AMB	0.125–2				1	1			1					
	VRC	0.03–0.25		1		1	1								
	ISA	0.06–0.5			1	1		1							
	POS	0.015–0.06	1		2										
	ITC	0.03–0.06		1	2										
	FLU	2–64								1				1	1
	CAS	8										3			
	MFG	8										3			
	AFG	8										3			
	5-FC	4–64									2				1
Exophiala jeanselmei (1)	AMB	4									1				
	VRC	1							1						
	ISA	8										1			
	POS	0.125				1									
	ITC	0.25					1								

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TABLE 2 (Continued)

Order and species (no. of isolates)	Drugs[a]	MIC[b]/MEC[c] range (μg/ml)	No. of isolates with MIC/MEC (μg/ml) of:												
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64
	FLU	64													1
	CAS	8										1			
	MFG	8										1			
	AFG	8										1			
	5-FC	64													1
Rhinocladiella mackenziei (1)	AMB	4									1				
	VRC	0.5						1							
	ISA	1							1						
	POS	0.125				1									
	ITC	0.25					1								
	FLU	>64													1
	CAS	2								1					
	MFG	4									1				
	AFG	4									1				
	5-FC	>64													1
Hysteriales (3)															
Rhytidhysteron spp. (3)	AMB	0.5–1						1	2						
	VRC	0.25–0.5					2	1							
	ISA	0.125–0.25				1	2								
	POS	0.25–0.5					2	1							
	ITC	0.06–0.125			1	2									
	FLU	64													3
	CAS	0.06–0.125			2	1									
	MFG	0.03–0.125		1	1	1									
	AFG	0.015–0.125	1		1	1									
	5-FC	64													3
Capnodiales (3)															
Cladosporium cladosporioides (3)	AMB	0.06–8			1			1				1			
	VRC	0.03–0.5		1		1		1							
	ISA	0.015–0.06	1		2										
	POS	0.03–0.06		2	1										
	ITC	0.03–0.06		1	2										
	FLU	1–64							1						2
	CAS	1–8							1	1		1			
	MFG	0.5–8						1	1			1			
	AFG	0.5–8						1		1		1			
	5-FC	0.5–64						1							2
Botryosphaeriales (2)															
Lasiodiplodia theobromae (1)	AMB	0.5						1							
	VRC	2								1					
	ISA	8										1			
	POS	8										1			
	ITC	16											1		
	FLU	64													1
	CAS	1							1						
	MFG	0.5						1							
	AFG	1							1						
	5-FC	64													1
Lasiodiplodia parva (1)	AMB	0.5						1							
	VRC	1							1						
	ISA	8										1			
	POS	8										1			
	ITC	16											1		
	FLU	64													1
	CAS	2								1					
	MFG	0.25					1								
	AFG	0.125				1									
	5-FC	64													1

(Continued on following page)

TABLE 2 (Continued)

Order and species (no. of isolates)	Drugs[a]	MIC[b]/MEC[c] range (μg/ml)	No. of isolates with MIC/MEC (μg/ml) of:												
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64
Microascales (1)															
Thielaviopsis ethacetica (1)	AMB	4									1				
	VRC	0.25					1								
	ISA	0.25					1								
	POS	0.06			1										
	ITC	1							1						
	FLU	1							1						
	CAS	2								1					
	MFG	2								1					
	AFG	8										1			
	5-FC	64													1
Sordariales (2)															
Chaetomium globosum (1)	AMB	0.5						1							
	VRC	0.25					1								
	ISA	1							1						
	POS	0.5						1							
	ITC	0.25					1								
	FLU	64													1
	5-FC	64													1
Madurella mycetomatis (1)	AMB	0.25					1								
	VRC	1							1						
	ISA	0.25					1								
	POS	0.25					1								
	ITC	1							1						
	FLU	>64													1
	CAS	4									1				
	MFG	2								1					
	AFG	2								1					
	5-FC	>64													1

[a]ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POS, posaconazole; AMB, amphotericin B; CAS, caspofungin; MFG, micafungin; AFG, anidulafungin; 5-FC, flucytosine.
[b]MICs taken for azoles and AMB.
[c]Minimum effective concentrations taken for echinocandins.

in a CMB case due to *Rhytidhysteron* spp. Notably, in a single case of eumycetoma, the etiologic agent, *M. mycetomatis*, was probably introduced by an intramuscular injection in the gluteal region given in rural settings (Fig. 2B and C). Fungal osteomyelitis was diagnosed in three patients. Two of these were due to *C. bantiana*, and in one case *Corynespora* spp. was the etiologic agent. Of these, one case had diabetes mellitus with chronic kidney disease as a risk factor, and in two other cases patients had trauma to the left knee and foot, respectively.

The most common underlying condition in 7 brain abscess cases diagnosed in this series was the immunocompromised state of the patients. Renal transplantation was noted in three cases of brain abscess due to *C. bantiana*, and one of the cases had coexisting HIV and hepatitis C virus (HCV) infections. Also, two cases of *C. bantiana* and *Fonsecaea monophora* brain abscess had hepatocellular carcinoma and acute promyelocytic leukemia, respectively. In one case of *C. cladosporioides*, the patient was on antitubercular therapy and also had a long duration of fungal sinusitis. Interestingly, a case of *R. mackenziei* brain abscess had a history of trauma on the arm followed by development of CNS symptoms. In the present series the majority of cases of ABPM diagnosed had precipitins and specific IgE positive for *A. alternata* ($n = 6$) and species of *Curvularia*. Further, three cases of lung PHM were observed. The first case was an elderly male with kidney transplantation, and a chest computed tomography scan demonstrated a cavitating lesion in the right lower lobe. An invasive infection was confirmed by the presence of melanized fungal elements in the biopsy tissue of the lung, as well isolation of *C. globosum* from BAL fluid. The second case was a 12-year-old boy with cystic fibrosis who presented with focal pulmonary consolidation, which was

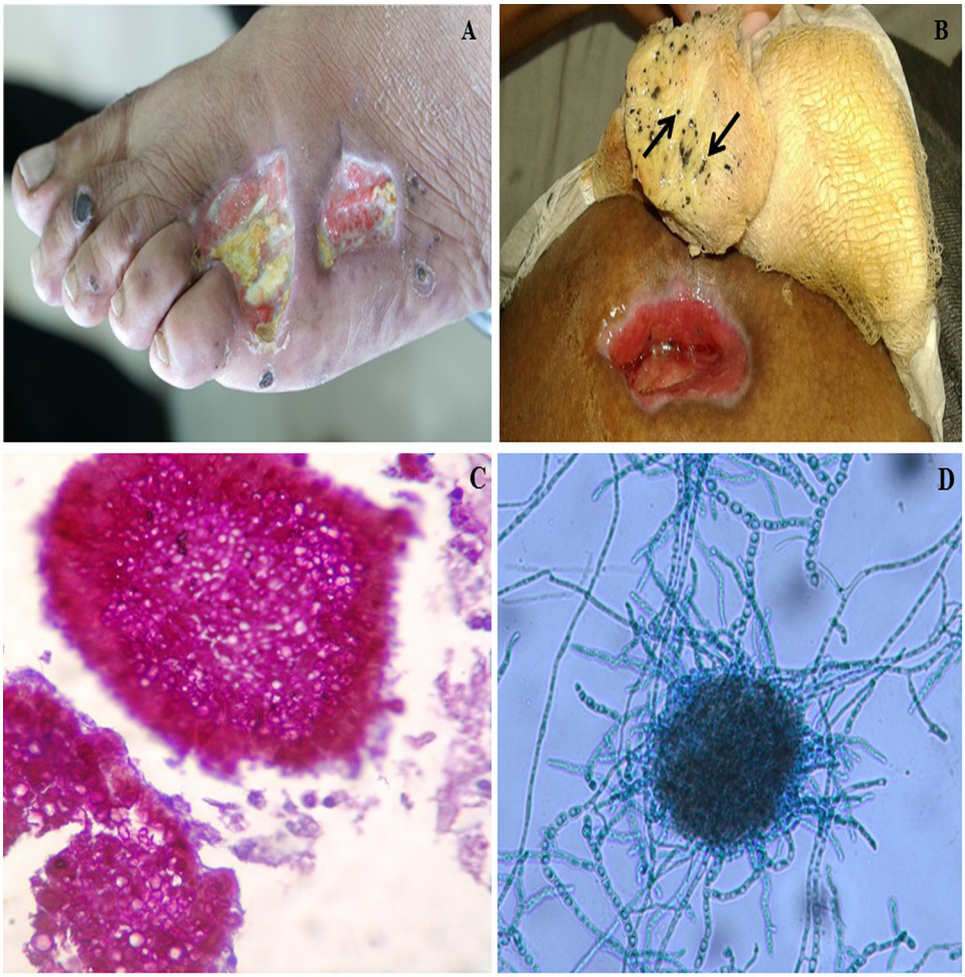

FIG 2 (A) Subcutaneous ulcer of the foot due to *A. alternata*. (B) Mycetoma of the buttock due to *M. mycetomatis* with black grains (arrow). (C) Section stained with periodic acid-Schiff stain (PAS) showing localized abscesses containing granules composed of a center showing interlacing hyphae embedded in a brownish matrix surrounded by eosinophilic, clublike Splendore-Hoeppli material (radially arranged hyphae; 200× PAS). (D) Slide culture of *R. percutanea* on PDA showing pycnidia at 7 days of incubation (40×).

empirically treated with antibiotics for bacterial pneumonia without improvement. An invasive infection was confirmed by a biopsy specimen and culture of BAL fluid with isolation of *E. dermatitidis*. Further, 80% of cases of keratitis were observed in patients with an agricultural background and reported trauma with vegetable matter while working in the fields.

ITC (200 to 400 mg/day) therapy, ranging from 6 to 18 months, along with partial surgical debridement, was administered in 10 (90%) cases of subcutaneous PHM. In three cases of PHM with cystic and nodular lesions, a complete surgical excision without antifungal therapy was the treatment. A combination therapy with ITC and terbinafine was required to successfully treat a case of mycetoma along with surgical debridement. All cases of CBM were treated with long-term ITC therapy without surgical debridement. The brain abscess was initially treated with AMB deoxycholate for 14 days and VRC at 400 mg/day for 6 to 12 months. A high mortality of 60% was observed in these cases. In AFRS cases, two-thirds of the patients had undergone sinus debridement. Also, cases of ABPM were treated with corticosteroids, administered systemically or locally. Two cases of pulmonary PHM were successfully treated with antifungal VRC therapy for a period of 2 months, whereas one patient died following the diagnosis. Osteomyelitis cases were the most refractory to treatment, which included repeated surgical resection and multiple courses of antifungals, including VRC or ITC for periods of up to 1 year.

DISCUSSION

The present study reports a broad range of MF species characterized by a polyphasic approach for a spectrum of clinical entities diagnosed in 19 centers in India. To the best of our knowledge, this is the largest case series of PHM to date where a MALDI-TOF MS approach was used successfully to identify diverse MF. In the present study, the sequence-based approach yielded 90% species identification of the isolates using ITS, LSU, and *β-tubulin* markers. Nevertheless, 4 isolates of the genera *Corynespora* and *Rhytidhysteron* could not be resolved to the species level by ITS sequence analysis due to insufficient information in the current GenBank database. Previously, ITS sequencing for *Curvularia* species demonstrated only 72.3% species identification because of putative new species in this genus (15). Further, 26% ($n = 19$) of isolates of *Alternaria*, *Curvularia*, *Corynespora*, *Fonsecaea*, *Rhytidhysteron*, *Chaetomium*, and *Phialemonium* could only be assigned to the genus level by LSU. Previously, the LSU domain was reported not to be useful for identification of some pathogenic or saprophytic *Phialophora* and *Cladosporium* species which have identical sequences or exhibited interspecies sequence diversity of less than 0.5% (16). Considering high species identification rates were observed using ITS sequencing in this study, ITS analysis could supplement traditional morphological methods in the identification of MF species from clinical specimens encountered in microbiology laboratories.

Mold identification using MALDI-TOF MS is more difficult than bacterial and yeast identification due to variable incubation time of slow/fast-growing molds of different genera/species. In addition, extensive variability in morphological characteristics also hinders the acquisition of reproducible spectra (17, 18). To minimize the effect of growth conditions on the production of uniform mycelium by fungi, Bruker Daltonics launched, in 2012, an additional spectral library, the Filamentous Fungi Library 1.0, that was constructed using 24- to 48-h-old liquid cultures for complete protein extraction. This library represents the first commercial database of Bruker Daltonics for the identification of molds grown in liquid media. Interestingly, since 2011 only one study utilized liquid cultivation with ethanol-formic acid extraction for sample preparation for identification of filamentous fungi by a MALDI Biotyper (19). Although protein extraction from cultures grown on solid media simplifies preanalytical processes and reduces the identification time, nonetheless, in our experience excellent identification was observed with cultures grown in liquid media. Considering that MF culture growth for up to 1 week is required for DNA extraction for molecular methods, MALDI-TOF MS yielded results with the liquid culture protocol in 48 to 96 h. The isolates belonging to the order Chaetothyriales grown in liquid media for 72 to 96 h were easily identified by MALDI-TOF MS depending on availability in the database.

The other issue regarding mold identification by MALDI-TOF MS is the reference database provided with each commercial MALDI-TOF MS platform, which may not be sufficient for routine analyses. One of the most clinically comprehensive mold databases, developed recently by Lau et al. and known as the NIH (National Institutes of Health, Bethesda, MD) mold database, yielded species-level identification for 88.9% (370/421) of clinical isolates tested, whereas the Bruker Biotyper library alone (version 3.3.1.) identified only 3 of 421 isolates. Lau et al. also created an in-house database for large numbers of MF ($n = 70$) spanning 20 genera and 30 species (10). Notably, compared to Lau et al., in the present study a higher isolate identification rate of 32% (23/72) was obtained with the commercial database, probably due to the usage of liquid mold cultures that Bruker utilized for database construction. Furthermore, 100% species-level identification was achieved by an in-house database creation of 20 species of MF analyzed in this study.

In the present study, rare MF were detected under several clinical conditions. *Roussoella percutanea* is a novel opportunistic pathogen reported in 2014 as an etiologic agent of subcutaneous infection in immunosuppressed patients (20, 21). Also, in the present study the first case of keratitis due to *R. percutanea* was observed. Further, *L. parva*, which had not been previously reported from human infections, was found to be the etiologic

agent of postoperative wound infection in a renal transplant patient, although other species, such as *L. theobromae*, detected as agents of keratitis in this study, have previously been documented as agents of keratitis and subcutaneous infections (22–25). Interestingly, fungal osteomyelitis of the foot due to *C. bantiana* and *Corynespora* spp. represent rare fungal infections in this series (26–28).

The present study represents one of the largest data sets of antifungal susceptibility patterns of many diverse MF. Considering that phylogenetically diverse MF were tested in this study, marked MIC variations at the genus and species levels were observed. Notwithstanding this finding, limited MIC and clinical outcome data with regard to MF hinders determination of epidemiological cutoff values and breakpoints for this class of molds. Nevertheless, low MIC values of POS ($\leq$1 μg/ml) and VRC ($\leq$2 μg/ml) were recorded in 96% and 95% of isolates. Previously, Ben Ami et al. reported that POS had low MICs against MF and is also effective in an animal model of PHM (2, 29). Also, echinocandins had high MECs ($\geq$2 μg/ml) in 39% of isolates, mainly among the genera *Medicopsis*, *Exophiala*, and *Cladophialophora*. Notably, high MICs of at least one drug were noted in 53% ($n = 30$) of the isolates. Finally, MF pose diagnostic and management challenges and are increasingly reported both in healthy and immunosuppressed hosts (30).

MATERIALS AND METHODS

Clinical specimens and selection of fungal isolates. A total of 13,494 clinical samples collected from 19 hospitals in India and referred to the Department of Medical Mycology, Vallabhbhai Patel Chest Institute (VPCI), Delhi, India, were processed for fungal culture during 2012 to 2015. Overall, during a 4-year study period, 718 (5.3%) clinical specimens were positive for MF. Of these, 72 isolates (10%) were selected for the analyses based on the histopathologic biopsy specimen confirmation of the clinical entities or their isolation from sterile sites. Further, isolates cultured from respiratory specimens were included based on repeated isolation from bronchoalveolar lavage (BAL) specimen/sputum or fine-needle aspiration biopsy (FNAB) specimens of lung and correlation with clinical and radiologic findings. The MF implicated in diagnosed cases of allergic bronchopulmonary mycoses (ABPM) were based on de Shazo and Swain (31). Proven infection was diagnosed according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria with isolates recovered from skin/tissue biopsy specimen and abscess drainage (32). Data from patients' medical records, including demographic characteristics, underlying conditions, nature, and results of imaging and histopathologic studies, treatment, and patient outcomes were obtained.

Morphological examination. All specimens were processed for direct microscopic examination by 10% potassium hydroxide (KOH)–Blankophor staining. They were inoculated on two sets each of Sabouraud's dextrose agar (SDA), one set containing gentamicin and chloramphenicol and the other containing cycloheximide (0.05%), and were incubated at 28°C and 37°C for 2 to 3 weeks. Preliminary macroscopic phenotypic identification of fungal isolates was done on 2% malt yeast extract agar (MEA), oatmeal agar (OA), and potato dextrose agar (PDA) incubated at 28°C for 14 days. Slide cultures prepared on the above-mentioned media were observed microscopically mounted in lactophenol cotton blue.

Molecular identification and phylogenetic analysis. Molecular identification of isolates was performed by sequencing the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) and the D1/D2 domain of the large subunit rDNA (28S), while a single isolate that failed to amplify with the above-mentioned loci was identified by *β-tubulin* gene sequencing. Mycelium was harvested from 7- to 14-day-old pure cultures on SDA at 28°C and then ground in the presence of liquid nitrogen and extraction buffer (0.2 M Tris-HCl, 10 mM EDTA, 0.5 M NaCl, 1% SDS) in a mortar and pestle, followed by phenol, chloroform, and isoamyl alcohol (25:24:1) extraction and ethanol precipitation. The extracted DNA was amplified using the ITS-1 (5′-TCCGTAGGTGAACCTTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTG ATATGC-3′) primers (33), NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCA AGACGG-3) primers (34), and Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGT AGTGACCCTTGGC-3′) primers (35). DNA sequencing was performed using the respective primers for PCR at a 0.5 μM concentration. All sequencing reactions were carried out in a 10-μl reaction volume using the BigDye Terminator kit, version 3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations and analyzed on an ABI 3130XL genetic analyzer (Applied Biosystems). ITS, D1/D2, and *β-tubulin* sequences were subjected to BLAST searches at GenBank (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Sequence-based species identification was defined by $\geq$99% sequence similarity with $\geq$99% query coverage. Genus identification was defined by 93 to 98% sequence similarity with $\geq$95% query coverage. Further, a neighbor-joining (NJ) tree based on aligned ITS gene sequences with 2,000 bootstrap replications was constructed using MEGA version 6 (36). The sequences of the type/reference strains of various melanized fungi were retrieved from GenBank and included for the phylogenetic analysis.

AFST. Antifungal susceptibility testing (AFST) was carried out using the Clinical and Laboratory Standards Institute broth microdilution method (CLSI-BMD), using the M38-A2 guidelines with minor

modifications (37). The isolates were grown for 2 to 3 weeks on PDA at 28°C, and conidial inocula were prepared in saline containing Tween 80 by gently scraping the surface of mature colonies with a sterile cotton swab moistened with sterile physiological saline and allowed to settle. The suspension was vortexed and adjusted to an optical density at 530 nm of 0.09 to 0.1 (2×10^5 to 5×10^5 conidia/ml) for sporulating molds, while for slowly sporulating to nonsporulating molds the modified CLSI M38-A2 method was followed. The modification included adjustment of inocula at higher optical density of 0.18 to 0.2 (1×10^6 to 2×10^6 hyphal fragments/ml). Antifungals tested included amphotericin B (AMB; Sigma-Aldrich, Germany); azoles, *viz*., itraconazole (ITC; Lee Pharma, Hyderabad, India), voriconazole (VRC; Pfizer Central Research, Sandwich, Kent, United Kingdom), posaconazole (POS; Merck, Whitehouse Station, NJ, USA), isavuconazole (ISA; Basilea Pharmaceutica International AG, Basel, Switzerland), and fluconazole (FLU; Sigma-Aldrich); echinocandins, *viz*., caspofungin (CFG; Merck), micafungin (MFG; Astellas Toyama Co. Ltd., Japan), and anidulafungin (AFG; Pfizer); and flucytosine (5-FC; Sigma-Aldrich). Drug-free and mold-free controls were included, and microtiter plates were incubated at 35°C. CLSI-recommended control strains of *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were included for every batch of isolates tested each day. MIC endpoints for all of the drugs except echinocandins were defined as the lowest concentration that produced complete inhibition of growth as read visually at 72 to 96 h for azoles, AMB, and 5-FC. Minimum effective concentrations (MEC) of echinocandins were defined as the lowest drug concentration that allowed the growth of small, rounded, and degenerated hyphae observed after 48 h.

MALDI-TOF MS identification and in-house database creation and validation. (i) Strain identification. Fungal isolates were identified by a MALDI-TOF MS Biotyper OC, version 3.1 (Bruker Daltonics, Bremen, Germany), using the ethanol-formic acid extraction method. Isolates were grown in Sabouraud's glucose broth (SGB) for 24 to 36 h at 28°C in a tube rotator. Briefly, 1 ml of culture was centrifuged at 20,800 $\times$ *g* for 2 min, followed by washing twice with 1 ml of deionized water. Further, the pellet was dissolved in 300 μl of deionized water, and 900 μl of absolute ethanol was added followed by vortexing and centrifugation at 20,800 $\times$ *g*. The pellet was air-dried and suspended in 50 μl of formic acid (70% [vol/vol]) (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 2 to 3 min at room temperature. An equal volume of acetonitrile (Sigma-Aldrich) was added, followed by vortexing and subsequent incubation for 4 to 5 min and centrifugation. Supernatant (1 μl) was applied to the target plate and air dried at room temperature. For isolates belonging to the order Chaetothyriales, a slightly modified protocol was used for protein extraction. The isolates were grown in SGB for an extended period of up to 96 h and were treated with ethanol and water twice with vortexing and incubation for 10 min at room temperature. This was followed by formic acid incubation for 15 to 20 min and subsequent incubation after addition of acetonitrile for the same time. The succeeding protocol for all of the isolates was as follows. The air-dried spot was overlaid with 1 μl of saturated α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics) dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid matrix solution. The spectra were recorded in the linear positive mode at a laser frequency of 20 Hz within a mass range of 2,000 to 20,000 Da. A total of 240 laser shots in six-shot steps from different positions of the target spot were collected and analyzed for each spectrum. The spectra were analyzed using the flexControl 3.1 (Bruker Daltonics) and MALDI Biotyper OC, version 3.1, softwares.

(ii) In-house database creation and validation. Main spectrum (MSP) was created for those MF that were not available in the current Bruker database (updated in September 2016) by using the manufacturer's protocol as described above. Each isolate was processed in triplicate, and eight spots were applied to the target plate from each replicate, corresponding to 24 spots for each isolate. The raw spectra of at least 20 spots of each isolate were further processed using Biotyper 3.1 software. To validate the in-house database, CBS reference strains (Table 1) and molecularly characterized reference strains previously stocked in the VPCI culture collection were selected for the corresponding species and subcultured from the glycerol stocks. At least 2 to 4 strains available in the stock culture collection, except *Lasiodiplodia parva* and *Thielaviopsis ethacetica*, were used for validation and processed as described above. A score of $\geq$2 was used for valid species identification.

Accession number(s). Sequences were deposited in GenBank under accession no. KY273520, KY303834 to KY303899, and KY310616 to KY310664.

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